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☐ *Additional inventors are being named on the ___ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

Mosquito Odorant Receptor and Method For Screening Chemicals Against Chemoreceptors

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Respectfully submitted,

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ABSTRACT& CLAIM

Mosquito Odorant Receptor and Method For Screening Chemicals Against Chemoreceptors

John R. Carlson & Elissa A. Hallem

Anopheles mosquitoes transmit malaria and are responsible for the death of over one million people each year. Female *Anopheles* mosquitoes locate their human hosts primarily through olfactory cues, yet the molecular mechanisms underlying this process are unknown. The identification of receptors for human odorants may be useful in developing new insect repellents and traps for the control of *Anopheles* and other insect pests. Here we show that AgOr1, a female-specific member of a family of candidate *Anopheles gambiae* odorant receptors, responds to a component of human sweat. We expressed a member of the *AgOr* gene family, *AgOr1*, in *Drosophila*. *AgOr1* was expressed in a screen consisting of a *Drosophila* olfactory receptor neuron that lacks odor response due to the deletion of its endogenous receptor genes, *Or22a* and *Or22b*, using an *Or22a* promoter and the *GAL4-UAS* system. The odor response of the neuron was assayed by single-unit electrophysiology.

We Claim:

1. Anopheles gambiae odorant receptors that respond to specific chemical components.
2. A screening method for identifying chemicals that bind to and/or stimulate specific chemoreceptors primarily in insects.

A Mosquito Odorant Receptor for a Component of Human Sweat

Anopheles mosquitoes transmit malaria and are responsible for the death of over one million people each year. Female *Anopheles* mosquitoes locate their human hosts primarily through olfactory cues[1], yet the molecular mechanisms underlying this process are unknown. The identification of receptors for human odorants may be useful in developing new insect repellents and traps for the control of *Anopheles* and other insect pests. Here we show that AgOr1, a female-specific member of a family of candidate *Anopheles gambiae* odorant receptors[2,3], responds to a component of human sweat.

We expressed a member of the *AgOr* gene family, *AgOr1*, in *Drosophila*. *AgOr1* was expressed in a *Drosophila* olfactory receptor neuron that lacks odor response due to the deletion of its endogenous receptor genes, *Or22a* and *Or22b,* using an *Or22a* promoter and the *GAL4-UAS* system[4]. The odor response of the neuron was assayed by single-unit electrophysiology.

We found that AgOr1 confers a strong response to the odorant 4-methylphenol (Fig. 1, top panel). 4-methylphenol is a component of human sweat that has been shown to elicit an electrophysiological response from the antenna of female *An. gambiae*[5], suggesting a role for AgOr1 in the anthropophilic host-seeking behavior of this mosquito. Consistent with this hypothesis, *AgOr1* is expressed specifically in the olfactory tissue of female but not male mosquitoes, and its expression is down-regulated following a blood meal[2]; host-seeking behavior of these mosquitoes is also female-specific and reduced by blood-feeding[6]. 4-methylphenol has also been shown to increase the effectiveness of traps for the tsetse fly, *Glossina morsitans morsitans*[7].

We then tested a second *AgOr* gene, *AgOr2*, and found a different odor response spectrum (Fig. 1, center panel). In contrast to *AgOr1, AgOr2* confers a strong response to 2-methylphenol, but not to 4-methylphenol.

These results demonstrate that *AgOr* genes encode odorant receptors, and suggest that the female-specific receptor *AgOr1* plays a role in the host-seeking behavior of *An. gambiae*. The ability of mosquito odorant receptors to function in *Drosophila*, in the absence of other mosquito-specific proteins, also suggests a broad compatibility between odorant receptors and olfactory receptor neurons of different species, and demonstrates the utility of the fruit fly as an *in vivo* model system for the study of odorant receptors derived from less genetically tractable insect species. The identification of receptors for particular human odorants suggests their use in screening for ligands that activate or inhibit these receptors. Some such ligands may be useful as attractants in traps, and others may be useful as repellents.

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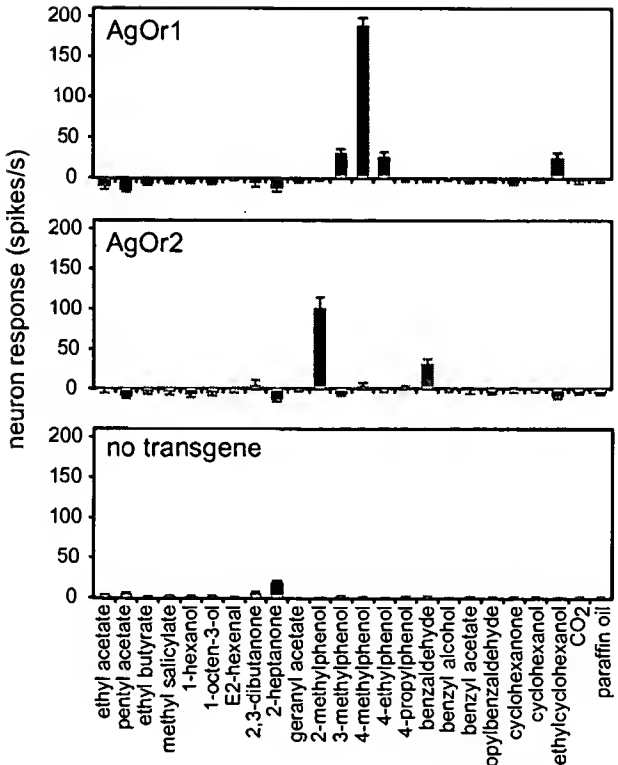
Fig. 1. Identification of a mosquito odorant receptor that responds to a component of human sweat by expression in a *Drosophila* olfactory receptor neuron.

Odor response spectrum conferred by AgOr1 (top panel) and AgOr2 (center panel) to a *Drosophila* olfactory neuron carrying a deletion of its endogenous receptor genes (lower panel). Transgenic flies were of the genotype w^{1118}; *Δhalo/Δhalo*; *UAS-AgOr/Or22a promoter-Gal4*[4,8]. Single-unit recordings were obtained as in Dobritsa *et al.*[4] from animals aged <1 wk. Liquid odors were diluted 10^{-4} in paraffin oil, and solid odors were diluted 0.2 mg/ml in paraffin oil. CO_2 was administered as in Dobritsa *et al.*[4]. Responses were quantified by subtracting the number of spikes in 500 ms of spontaneous activity from the number in the 500 ms after the onset of odor stimulation. n = 12 and error bars = SEM. *AgOr* cDNA clones were from *An. gambiae* sensu stricto (G3 strain); embryos were kindly provided by Dr. Mark Benedict (CDC, Atlanta, GA) and subsequently reared as described[2].


AgOr1
AgOr2
no transgene
neuron response (spikes/s)
200
150
100
50
0
ethyl acetate
pentyl acetate
ethyl butyrate
methyl salicylate
1-hexanol
1-octen-3-ol
E2-hexenal
2,3-dibutanone
2-heptanone
geranyl acetate
2-methylphenol
3-methylphenol
4-methylphenol
4-ethylphenol
4-propylphenol
benzaldehyde
benzyl alcohol
benzyl acetate
4-isopropylbenzaldehyde
cyclohexanone
cyclohexanol
4-methylcyclohexanol
CO2
paraffin oil

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Integrating the Molecular and Cellular Basis of Odor Coding in the *Drosophila* Antenna

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Summary

We investigate how the molecular and cellular maps of the *Drosophila* olfactory system are integrated. A correspondence is established between individual odor receptors, neurons, and odors. We describe the expression of the *Or22a* and *Or22b* receptor genes, show localization to dendritic membranes, and find sexual dimorphism. Or22a maps to the ab3A neuron, which responds to ethyl butyrate. Analysis of a deletion mutant lacking *Or22a*, along with transgenic rescue experiments, confirms the mapping and demonstrates that an *Or* gene is required for olfactory function in vivo. Ectopic expression of Or47a in a mutant cell identifies the neuron from which it derives and its odor ligands. Ectopic expression in a wild-type cell shows that two receptors can function in a single cell. The ab3A neuron does not depend on normal odor receptor gene expression to navigate to its target in the CNS.

Introduction

Animals are able to sense and discriminate among a remarkable number of odors (Hildebrand and Shepherd, 1997). Olfactory information is received and encoded by olfactory receptor neurons (ORNs). These neurons encode the quality and intensity of odors, as well as aspects of their spatiotemporal distribution. The code is in the form of action potentials and is based on the differential responses of ORNs to different olfactory stimuli. The signals generated by ORNs are transmitted to the brain, where processing takes place.

ORNs vary in their odor specificity, sensitivity, and response dynamics. The cellular basis of the odor code has been explored in detail in *Drosophila*, whose relatively simple olfactory system allows precise physiological measurements of individual ORNs in vivo (De Bruyne et al., 1999, 2001). Flies contain two olfactory organs, the antenna and the maxillary palp, which contain ~1200 and ~120 ORNs, respectively (Stocker, 1994; Shanbhag et al., 1999, 2000). These ORNs are compartmentalized in olfactory sensilla, which divide into morphologically distinct classes, including large basiconic sensilla, small basiconic sensilla, trichoid sensilla, and coeloconic sensilla. Each sensillum contains up to four neurons, whose activities can be defined by extracellular electrophysiological recordings (Clyne et al., 1997; De Bruyne et al., 1999, 2001).

In *Drosophila*, extensive recordings have revealed that ORNs fall into distinct functional classes based on their odor response spectra. Sixteen functional classes of ORNs, each with a unique response spectrum to a panel of 47 odors, were identified from recordings of antennal basiconic sensilla (De Bruyne et al., 2001). These ORNs exhibit diverse response dynamics, including excitatory and inhibitory responses, and various modes of termination kinetics. The 16 ORN classes are found in stereotyped combinations in seven functional types of basiconic sensilla, each mapping to a defined subregion of the antennal surface.

Functional differences among ORN classes are believed to arise from the expression of different odor receptors. A family of at least 60 seven-transmembrane-domain receptor genes, the *Or* genes, was discovered in *Drosophila* and proposed to encode odor receptors (Clyne et al., 1999a; Gao and Chess, 1999; Vosshall et al., 1999, 2000). Individual *Or* genes are expressed in different subsets of ORNs. A mutation that alters the expression of a subset of *Or* genes alters the odor specificity of a subset of ORNs (Clyne et al., 1999b), and direct evidence was recently found for the involvement of one *Or* gene in olfactory signaling (Störtkuhl and Kettler, 2001; Wetzel et al., 2001).

The isolation of *Or* genes and the functional identification of discrete ORN classes by physiological analysis now allows a critical problem to be explored in *Drosophila*: the integration of the molecular and cellular maps of the olfactory system. Here we demonstrate three means of mapping the receptor repertoire to the neuronal repertoire. Moreover, since the odor specificities of the neurons are defined, the results by extension map receptor space to odor space. This approach allows an integrated molecular and cellular definition of the basis of odor coding.

We demonstrate that individual receptors map to individual neuronal classes through a genetic and molecular analysis of two *Or* genes, *Or22a* and *Or22b*. We describe the expression of these genes by immunohistochemistry and show localization to dendritic membranes by immunoelectron microscopy. The Or22a receptor is mapped to the ab3A neuron, by using its promoter and the *GAL4-UAS* system (Brand and Perrimon, 1993) to drive expression of GFP or the cell death gene *reaper*, followed by physiological recordings from individual sensilla. We thereby link the Or22a receptor to the odor ethyl butyrate, to which ab3A is highly sensitive.

Analysis of a mutant lacking *Or22a*, together with rescue experiments using an *Or22a* transgene, confirm the

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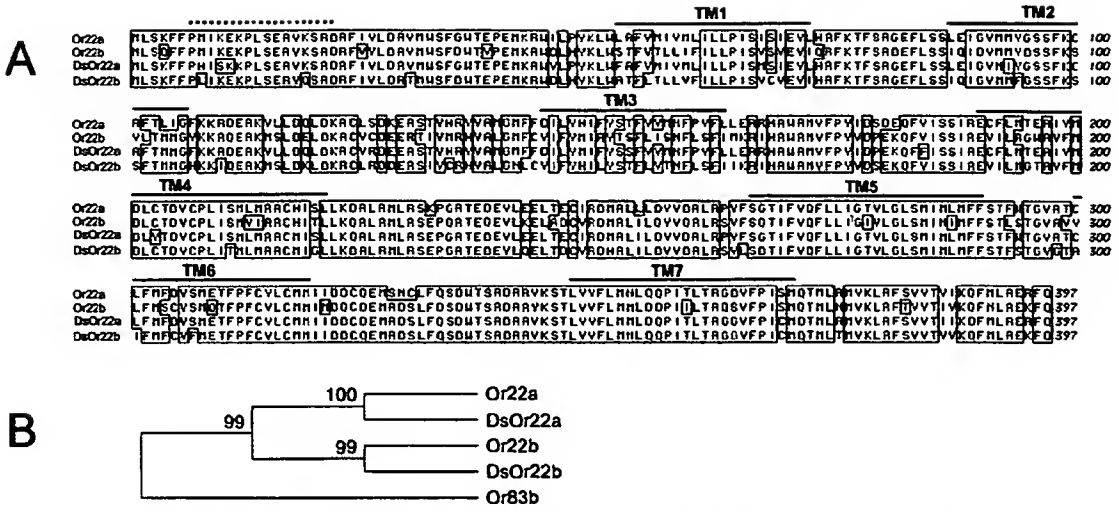

Figure 1. *Or22a* and *Or22b* and Their Orthologs from *Drosophila simulans*

(A) Amino acid alignments. Identical regions are shaded. The dotted line indicates the peptide used for antibody production. The approximate positions of the predicted transmembrane domains 1 through 7 (Clyne et al., 1999a) are labeled TM1-7. *Or22a* and *Or22b* are from *D. melanogaster*; *DsOr22a* and *DsOr22b* are from *D. simulans*.

(B) Phylogenetic relationship between *Or22a*, *Or22b*, *DsOr22a*, and *DsOr22b*, constructed using the neighbor-joining method. *Or83b* is used as an outgroup. Numerical values indicate bootstrap support for each node.

mapping of *Or22a* to the ab3A neuron. This genetic analysis provides direct evidence that an *Or* gene is required in vivo for normal odor detection.

We provide evidence that *Or22b* is coexpressed with *Or22a* in the same cell but that *Or22b* is neither necessary nor sufficient for ab3A function; rather, the broad response spectrum of the ab3A neuron is accounted for by a single receptor. Ectopic expression of another receptor, Or47a, in the mutant ab3A neuron is used to identify the ORN from which Or47a derives and to determine its odor specificity. These results show that the odor response spectrum of an ORN in *Drosophila* depends on the *Or* gene that it expresses. Expression of *Or47a* in a wild-type ORN shows that two receptors are able to function in a single cell.

Finally, we address the possibility of a developmental role for *Or* genes, a possibility that has not been systematically investigated by expression analysis and that can be rigorously determined only by functional analysis. We show that the ab3A ORN is able to navigate toward its target in the CNS if *Or22a* and *Or22b* are deleted or substituted by other receptor genes.

Results

Or22a/b Expression Is Sexually Dimorphic and Is Localized to Dendritic Membranes of ORNs

Or22a and *Or22b*, the first *Or* genes identified in our computational screen for *Drosophila* odor receptors (Clyne et al., 1999a), are tightly clustered, lying within 650 bp of each other in the genome. Clustering is common among *Or* genes, with more than one-third of the family members located in clusters of up to three genes. *Or22a* and *Or22b* are among the most closely related members of the family, showing 78% amino acid identity (Figure 1). The average identity among *Or* genes in a cluster is ~45%.

In order to test the hypothesis that *Or22a* and *Or22b* encode odor receptors, and to examine the relationship between Or expression and sensillum type, we sought to determine at high resolution the cellular and subcellular distribution of the Or22a and Or22b proteins. For this purpose we raised a polyclonal antibody against a 16 amino acid sequence common to the N terminus of both proteins (Figure 1A); this region was selected because it was predicted by bioinformatic analysis to generate effective antibodies. If Or22a and Or22b are in fact odor receptors, we would expect them to be localized to the dendritic membranes of ORNs. We also wished to determine whether the labeled sensilla all belonged to the same morphological type of olfactory sensilla. In situ hybridization with *Or22a* and *Or22b* probes had shown labeling of cells in the dorso-medial region of the antenna (Figure 2A) but was of insufficient resolution to determine with precision the morphological types of sensilla associated with the labeled cells.

The antibody stained a subset of the large basiconic sensilla in the dorso-medial region of the antenna (Figure 2B). Labeling is clearly visible in the sensillum shaft, where the dendrites of ORNs are located (Figure 2C). We did not observe labeling in cell bodies or axons. No labeling was detected in the maxillary palp, the other adult olfactory organ, or in the larval antenno-maxillary complex, which mediates both larval olfaction and taste.

Sexual dimorphism was observed: although both males and females showed similar spatial patterns of labeling, the number of labeled sensilla in females, 29 ± 2 (n = 13), was greater than that in males, 18 ± 2 (n = 10) ($p < 0.0001$; Table 1).

Immunoelectron microscopy confirmed that the labeled sensilla were large basiconic sensilla and allowed a precise definition of their morphological subtype, LB-I. Sensilla of this subtype are characterized by a relatively thin cuticle, and they contain two ORNs per sensillum

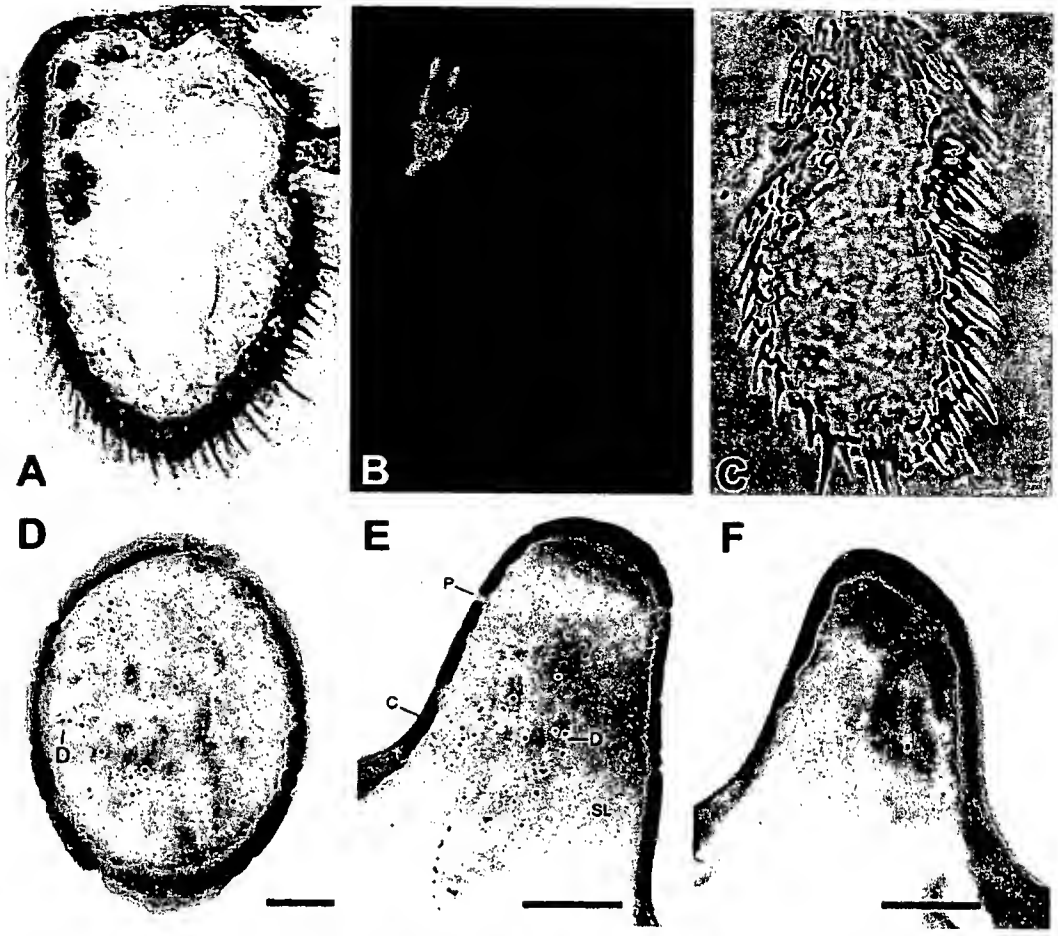

Figure 2. Expression of Or22a/b in the Dendrites of ORNs in Large Basiconica Sensilla

(A) In situ hybridization with an *Or22b* probe reveals RNA expression in a subset of cells in the dorso-medial area of the antenna.

(B) Fluorescent immunostaining of the antenna with the anti-22a/b antibody labels the shaft of a subset of large basiconic sensilla in the dorso-medial area. No signal was detected with preimmune sera or with antibodies preincubated with the peptide against which they were raised.

(C) Overlay of (B) with the corresponding bright-field image.

(D–F) Immunoelectron microscopy of large s. basiconica shows expression of Or22a/b proteins in dendritic membranes. Scale bars equal 1 μm.

(D) Crosssection labeled with anti-22a/b antibody. Granules of immunogold are visible in the dendrites (labeled D).

(E) Longitudinal section labeled with anti-22a/b antibody. Labels indicate the following: C, cuticle; D, dendrite; P, pore; and SL, sensillum lymph.

(F) An example of a different morphological subtype of s. basiconica, which shows no labeling with anti-22a/b antibody.

(Shanbhag et al., 1999). In agreement with our findings, this subtype exhibits sexual dimorphism, with 33 LB-I sensilla present in females and 19 in males (Shanbhag et al., 1999). Immunoelectron microscopy further revealed that the label is distributed on the surfaces of dendrites (Figures 2D–2F), consistent with the expression of membrane receptors.

Or22a/b Map to a Unique Functional Type of Sensillum: ab3

Having determined that *Or22a/b* localize to a particular morphological type of sensillum, we next sought to localize them to a functional type of sensillum. A map of the seven well-characterized functional types of antennal basiconic sensilla is shown in Figure 3A. The different types show overlap in their distributions, but each type is restricted to a particular spatial domain on the antennal surface (De Bruyne et al., 2001). There are three functional types of large basiconic sensilla, ab1, ab2, and ab3, defined on the basis of electrophysiological recordings from the ORNs they contain. ab1 sensilla are easily recognized in such recordings because they contain four ORNs with distinct spike amplitudes or shapes, whereas ab2 and ab3 each house two ORNs (De Bruyne et al., 2001). All three sensillum types can be distinguished by measuring the response of their neurons to a panel of odors (Figure 3I). Throughout this study we have monitored ORN activity by recording action potentials, which provide a direct measure of ORN signaling.

Table 1. Number of Large Basiconic Sensilla in Different Morphological and Functional Classes

Large Basiconic Sensillum Types	Males	Females
anti-22a/b-positive	18 ± 2 (n = 10)[a]	29 ± 2 (n = 13)[a]
LB-I[b]	19	33
LB-II2[b]	24	23
LB-II4[b]	47	48
ab1[c]	45	ND
ab2[c]	27	ND
ab3[c]	18	ND

ND, not determined
[a]Mean ± SEM; complete series of sections were scored for each antenna.
[b]Morphological classes; LB, large basiconic sensilla; data from Shanbhag et al. (1999)
[c]Functional classes; data from De Bruyne et al. (2001)

To determine in which functional type or types of sensilla *Or22a/b* are expressed, we have made physiological recordings from live flies in which the *Or22a/b*-expressing sensilla are labeled with GFP. In brief, we generated two strains of transgenic flies in which the presumed promoters of *Or22a* or *Or22b* drive expression of the yeast transcription factor GAL4 (Brand and Perrimon, 1993), which in turn drives expression of GFP. We then recorded from GFP-labeled sensilla, which allows us to correlate a particular receptor gene with a particular sensillum type.

Specifically, to drive GAL4 under the control of the Or22a promoter, we isolated an 8.2 kb region upstream of the *Or22a* translational start codon and fused it to the coding sequence of *GAL4* to generate a construct we refer to as *22a-GAL4* (Figure 3B). To drive GAL4 under the control of the *Or22b* promoter, we isolated a 10.3 kb region upstream of *Or22b* to generate *22b-GAL4*. These 10.3 kb include the 8.2 kb upstream of *Or22a*, the *Or22a* coding sequence, and the intergenic region between *Or22a* and *Or22b* (Figure 3B). Flies carrying the *22a-GAL4* or *22b-GAL4* transgenes were crossed to flies carrying *UAS-GFP* to yield progeny in which GAL4 binds to a *UAS* and activates transcription of *GFP*. The particular GFP derivative we used was mCD8-GFP, which contains sequences of the mouse lymphocyte surface marker CD8 and which accordingly localizes to membranes (Lee and Luo, 1999). This derivative is hereafter referred to as "GFP" for simplicity.

We found that *22a-GAL4* drives expression of GFP in a subset of large basiconic sensilla in the dorso-medial region of the antenna (Figures 3C and 3D), and *22b-GAL4* shows an indistinguishable pattern (Figure 3E). To confirm that GFP expression recapitulates the endogenous Or22a/b expression pattern, we performed double-labeling experiments with anti-Or22a/b and antibodies directed against GFP. All sensilla expressing GFP were found also to express Or22a/b in the case both of the *22a-GAL4* driver (Figures 3F and 3G) and the *22b-GAL4* driver (not shown; see below).

The GFP-labeled sensilla are visible in live animals, thereby allowing us to distinguish them and record from them electrophysiologically. Recordings from labeled sensilla of *22a-GAL4; UAS-GFP* flies revealed that these sensilla house two neurons, as expected of ab2 or ab3, but not ab1. We tested the labeled sensilla with a diagnostic set of 11 odors that distinguish among the different types of large basiconic sensilla. We found that the sensilla labeled with *22a-GAL4* were homogeneous in their response spectrum. They contain an A neuron whose strongest responses are to ethyl butyrate, pentyl acetate, and ethyl acetate (like the A neuron of the ab3 sensillum) and a B neuron whose strongest responses are to heptanone, hexanol, and octenol (like the B neuron of the ab3 sensillum) (Figures 3H and 3I). In the *22b-GAL4; UAS-GFP* flies, the labeled sensilla were also homogeneous: they contained two ORNs each, and the ORNs yielded a response pattern similar to that of ab3 (not shown).

These results indicate that both *22a-GAL4* and *22b-GAL4* drive expression in the ab3 sensillum. We note further that the total number of ab3 sensilla estimated for the male antenna in the physiological studies of De Bruyne et al. (2001) is 18, a number that agrees well with the number of sensilla labeled in males with the anti-*Or22a/b* antibody, 18 ± 2, and the number of LB-I sensilla in males as determined in ultrastructural studies, 19 (Table 1; Shanbhag et al., 1999).

Although all sensilla expressing GFP reacted with the anti-*Or22a/b* antibody, some anti-*Or22a/b*-reactive sensilla did not show expression of GFP (24.6% for *22a-GAL4* [n = 264]; 9.6% for *22b-GAL4* [n = 209]). A simple interpretation of this observation is that these two GAL4 lines are not completely expressive, an interpretation we have also drawn from experience with several other *Or-GAL4* lines (D. Lessing and J.R.C., unpublished results). In support of this interpretation, we found no physiological differences between GFP^+ and GFP^- ab3 sensilla in either *22a-GAL4; UAS-GFP* or *22b-GAL4; UAS-GFP* lines.

Or22a/b Map to the ab3A Neuron

To increase the resolution of our mapping from sensillum type to neuron type, we adopted a modified strategy. We again used *Or* promoter-*GAL4* constructs and single-unit electrophysiology, but rather than using GAL4 to drive GFP, we used it to drive the cell death gene *reaper* (*rpr*). Specifically, we asked whether *Or22a-GAL4; UAS-rpr* or *Or22b-GAL4; UAS-rpr* antennae lacked a particular ORN.

Recordings from ab3 sensilla of *Or22a-GAL4; UAS-rpr* flies do not show the large spikes characteristic of the ab3A neuron (Figure 4A). By contrast, the small spikes characteristic of ab3B are present. To characterize the effect of *rpr* expression more fully, we tested both *Or22a-GAL4; UAS-rpr* and *Or22b-GAL4; UAS-rpr*

flies with the entire panel of odors (Figure 4B). In both genotypes, there is no response of the ab3A neuron to any tested odor. The ab3B neuron, however, responds strongly to all of the odors that elicit a response from a control line, *Or22a-GAL4; UAS-GFP*. The ORNs of the other types of large basiconic sensilla appeared normal in limited testing. These results indicate that both *Or22a* and *Or22b* drivers direct expression in the ab3A neuron.

We note with interest that for some odors, most notably pentyl acetate, mean response of the ab3B neuron in the *rpr* lines is greater than that in the control. Further investigation will be needed to establish whether this effect reflects an inhibitory role of the ab3A cell on the activity of the neighboring ab3B cell in wild-typs sensilla.

A Mutant Deleted for *Or22a* and *Or22b* Shows Abnormal Physiology of the ab3A Neuron

To investigate the function of the *Or22a* and *Or22b* genes directly, we used a synthetic deletion that removes both genes, a deletion referred to hereafter as Δ*halo*. Immunostaining of the mutant antenna with the anti-*Or22a/b* antibody reveals no labeling (Figure 5A).

Electrophysiological recordings from the large basiconic sensilla in mutant flies revealed that the ab3A neurons are unresponsive to all odors of our test panel: for all odors tested, the mean response is ≤18 spikes/sec (Figures 5E and 5F). By contrast, the ab3B neurons in the mutant sensilla show a response spectrum similar to that of wild-type ab3B neurons (Figure 5E), with the exception of an unexpectedly large response of the ab3B cell to pentyl acetate in the mutant, as observed in the *rpr* ablation experiments. All other neuronal classes in large basiconic sensilla appear normal as well, as judged by testing the four neuronal classes of ab1 (n = 11 sensilla; 44 ORNs total) and the two neuronal classes of ab2 (n = 10; 20 ORNs) with ths odors to which they respond most strongly (ethyl acetate, 2,3-dibutanone, CO_2, methyl salicylate, ethyl acetate, 1-hsxanol, and ethyl butyrate; see Figure 3I). Thus, deletion of *Or22a* and *Or22b* has a profound effect on the ab3A cell, consistent with the mapping of *Or22a* and *Or22b* to ab3A in the *rpr*-ablation experiments.

To test the possibility that ab3A expresses an additional odor receptor that functions independently of *Or22a* or *Or22b*, we challenged ab3 sensilla in the deletion mutant and in wild-type with odors of complex natural food sources: banana, orange, and apple. We found that these odor mixtures elicited strong activity in the wild-type from both ab3A and ab3B neurons, but in the mutant they elicit a response only from the ab3B neuron (not shown). Thus, in the absence of *Or22a* and *Or22b*, the ab3A cell does not respond to any of a wide variety of tested odors.

In addition to the severe loss of odor response in ab3A cells lacking *Or22a* and *Or22b*, we observed a second physiological phenotype: an abnormality in the temporal pattern of those spikes that are observed at low frequency in mutant ab3A cells. Although two of ten ab3A neurons examined in Δ*halo* were entirely silent, the others showed a low level of activity that consisted largely of bursts of action potentials. Bursts typically contained three or four action potentials, with an interspike interval of 14 ± 0.8 ms (n = 73; SEM). These bursts occurred at ~10 s intervals in the absence of odor stimulation. The frequency of bursts increased during responses of the neighboring ab3B neuron (Figure 5F), but the overall frequency of firing was still very low: for example, when ab3B was stimulated with 2-heptanone, we recorded 18 ± 3.9 (n = 10) impulses s^{-1} from ab3A.

Or22a, and not *Or22b*, Is Responsible for the Odor Sensitivity of ab3A

We have shown that a mutant deleted for *Or22a* and *Or22b* suffers a loss of odor response and abnormal firing of the ab3A neuron. The deletion, Δ*halo*, is a synthetic deficiency that combines $Df(2L)dp^{79b}$ and $Dp(2;2)dpp^{d21}$, thereby removing a fragment of ~100 kb in cytogenetic region 22A (M. Welte, personal communication; see Experimental Procedures). Do the physiological phenotypes we have documented arise from loss of *Or22a*, *Or22b*, both, or neither? This question is of special interest in light of the broad response spectrum of the ab3A neuron and the relatively high degree of sequence identity between *Or22a* and *Or22b*. We were especially interested in testing the hypotheses that (1) *Or22a* mediates response to a subset of the odors to which ab3A responds, while *Or22b* mediates response to a different subset; (2) *Or22a* and *Or22b* form an obligate heterodimer; (3) *Or22a* and *Or22b*, which are closely related, are functionally redundant; (4) one receptor mediates all the odor responses of ab3A.

To distinguish among these hypotheses, we carried out transformation rescue experiments with transgenic constructs carrying *Or22a*, *Or22b*, both, or neither. We reasoned that if each transgene rescued a portion of the response, then hypothesis 1 was likely correct; if rescue required cotransformation with both receptors, then hypothesis 2 was likely correct; if response were rescued by either receptor, then hypothesis 3 was likely correct; and if the response were rescued by one receptor, but not the other, then hypothesis 4 was likely correct.

Accordingly, we generated four transgenic constructs, which we refer to as $22a^{+}22b^{+}$, $22a^{+}22b^{-}$, $22a^{-}22b^{+}$, and $22a^{-}22b^{-}$. The $22a^{+}22b^{+}$ construct contains 12 kb of genomic DNA carrying wild-type copies of both *Or22a* and *Or22b* as well as all the upstream sequences contained in the *22a-GAL4* and *22b-GAL4* constructs. The $22a^{+}22b^{-}$ construct is identical, except that stop codons were inserted into the predicted transmembrane domain 1 (TM1) of *Or22b*. In the $22a^{-}22b^{+}$ construct, we introduced a frameshift mutation to create a stop codon in TM1 of *Or22a*. $22a^{-}22b^{-}$ contains both the *Or22a* and *Or22b* mutations. The four constructs were then introduced separately into Δ*halo* mutants, and transgenic lines were tested for electrophysiological responses to the diagnostic set of odors.

The $22a^{+}22b^{+}$ transgene rescued the activity of the ab3A neuron, indicating that the mutant phenotype is in fact caused by the absence of one or both *Or* genes (Figure 5G). As expected, the $22a^{-}22b^{-}$ construct did not rescue the phenotype. The $22a^{+}22b^{-}$ construct rescued the response of ab3A, to the same extent as $22a^{+}b^{+}$, as if $22a^{+}$ alone is sufficient to rescue. By contrast, the $22a^{-}b^{+}$ construct provided no appreciable rescue of the odor response in either males or females.

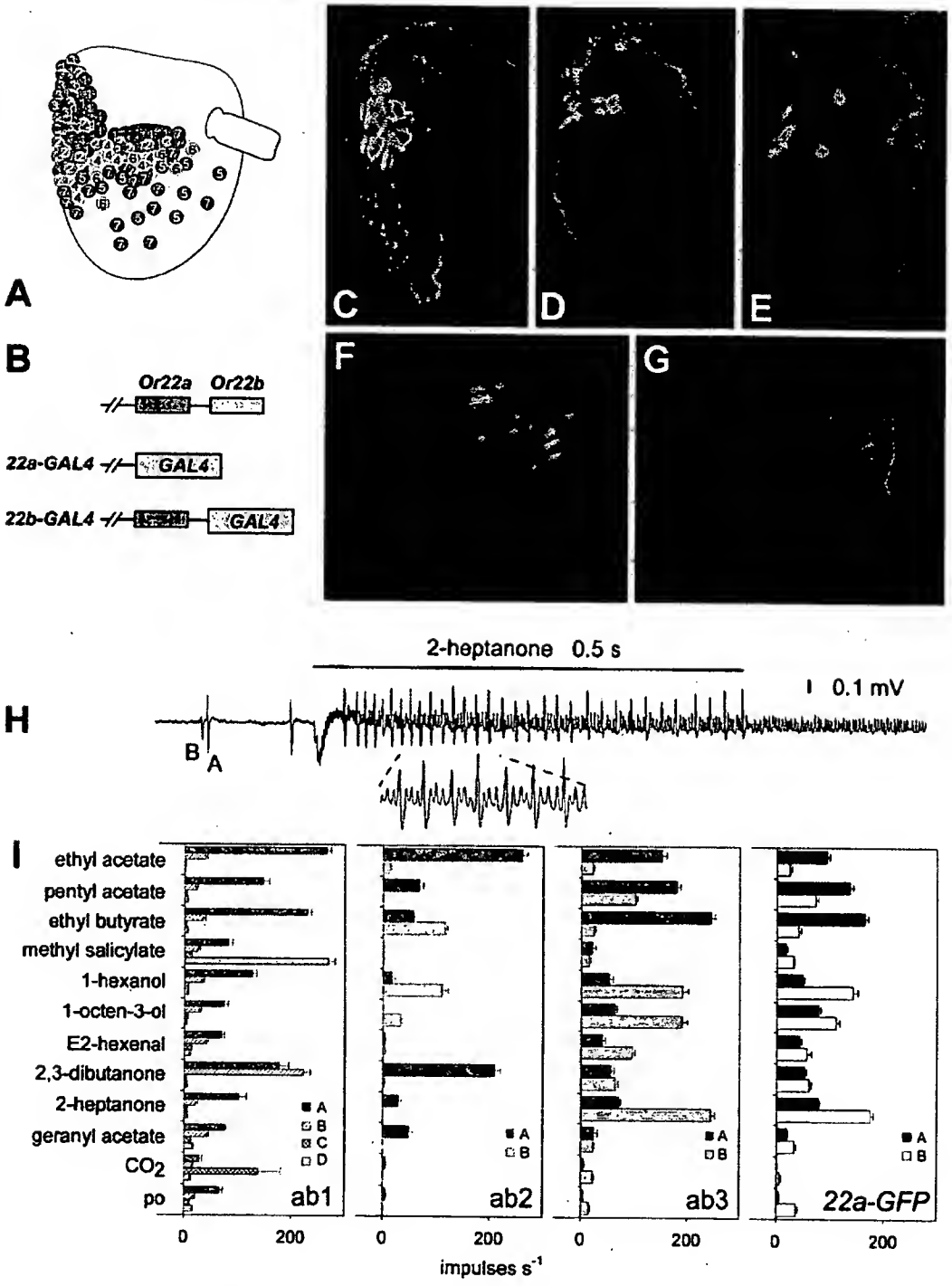

Figure 3. *22a-GAL4* and *22b-GAL4* Are Expressed in ab3 Sensilla

(A) Map of seven functional types of basiconic sensilla on the antenna. The number 3 indicates ab3 sensilla. Posterior face; dorsal is at top; medial is to the left. Adapted from De Bruyne et al., 2001.

(B) Structure of the *22a-GAL4* and *22b-GAL4* constructs. The *GAL4* coding sequences replace the coding sequences of the respective *Or* genes, and the constructs contain 8.2 and 10.3 kb DNA upstream of *Or22a* or *Or22b*, respectively.

(C and D) Confocal images of GFP expression driven by *22a-GAL4* in antennae of live flies (*UAS-GFP; 22a-GAL4*). Expression is restricted to the dorso-medial area. Dendrites, two cell bodies, and a short portion of an axon are apparent in (D).

(E) Expression of GFP driven by *22b-GAL4*. Dendrites are visible at left; two cell bodies appear near the center of the section.

(F and G) GFP expressed in *UAS-GFP; 22a-GAL4* flies localizes to Or22a/b-positive cells.

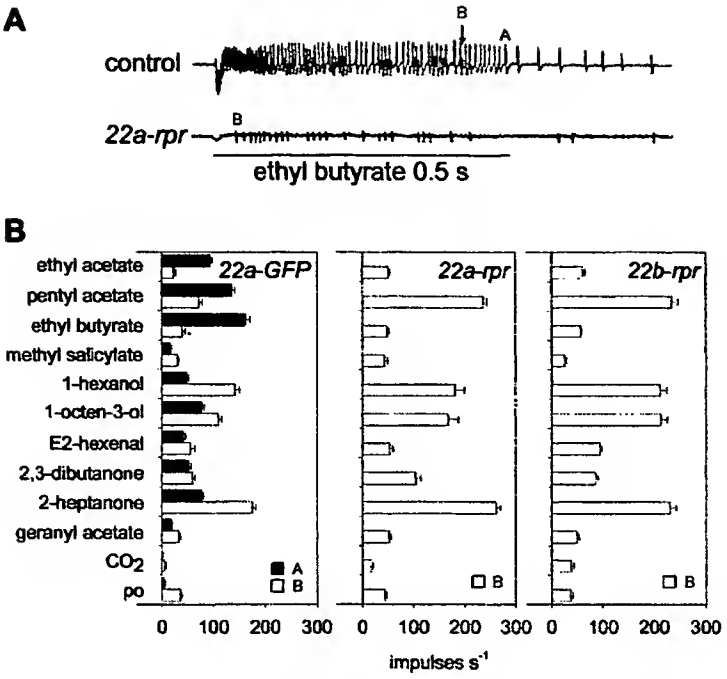

Figure 4. Ablation of the A Neuron in the ab3 Sensilla by the Cell Death Gene *rpr* Driven by *22a-GAL4* or *22b-GAL4*

(A) Recordings from ab3 sensilla in control flies (top trace, *UAS-GFP*; *22a-GAL4/+*) and from *22a-rpr* flies (bottom trace, *UAS-GFP*; *22a-GAL4/UAS-rpr*) in response to ethyl butyrate (horizontal bar). In the control, the A neuron (large spikes) is strongly stimulated by the odor, while the B neuron (small spikes) exhibits moderate activity. In the *22a-rpr* flies, only the small spikes corresponding to the B neuron are present.

(B) Response profiles of the ab3 sensilla from the *22a-GFP* control, *22a-rpr*, and *22b-rpr* flies to the diagnostic set of odors (n = 10–12). Only the activity of the B neurons is detectable in a large proportion of the sensilla from both *rpr*-expressing lines, and the responses of the ORNs in these sensilla are shown. A small fraction of ab3 sensilla were normal in these lines, presumably due to incomplete penetrance of *rpr* expression.

The bursting phenotype also was not rescued. Thus, rescue is provided only by those constructs containing an intact *Or22a* gene, suggesting that *Or22a* is necessary for rescue, with no rescue provided by the addition of *Or22b*. A caveat in this analysis is that little immunoreactivity was observed in the line carrying the $22a^{-}22b^{+}$ rescue construct, and we therefore sought to test the function of *Or22b* by other means.

To test further the roles of *Or22a* and *Or22b* in ab3A response, we expressed *Or22a* and *Or22b* in the *Δhalo* background using the *GAL4-UAS* system. Specifically, *Or22a* or *Or22b* was placed under the control of a *UAS* and expressed using the *22a-GAL4* driver. We found that the expression of *Or22a* by this method rescued the response of ab3A, while expression of *Or22b* did not, in either of two independent insertion lines, either in males (Figure 5G) or in females (not shown). Expression of *Or22b* in this neuron conferred no appreciable response to any of 87 tested odors, including a wide variety of alcohols, aldehydes, acetate esters, organic acids, ketones, and terpenes. Nor was the bursting phenotype affected by the introduction of *UAS-Or22b*. Expression of Or22b in neurons in the expected region of the antenna was observed in this line (Figure 5D), in a pattern comparable to that in other transgenic rescue lines (Figures 5B and 5C). The simplest interpretation of all the rescue results, taken together, is that a single *Or* gene, *Or22a*, is necessary and sufficient for the odor response of the ab3A neuron.

Is *Or22b* expressed in the wild-type antenna? First, *Or22b* probes, like *Or22a* probes, label the antenna by in situ hybridization (Figure 2A), although we cannot exclude the possibility that at least some of this labeling is due to crosshybridization to *Or22a* RNA. Second, we have found evidence for expression of *Or22b* in each of three independent preparations of antennal RNA from RT-PCR analysis, using multiple sets of primers, followed by sequence analysis. Finally, cDNA clones corresponding to both *Or22a* and *Or22b* have been isolated from an antennal/maxillary palp cDNA library (Vosshall et al., 1999). Thus, *Or22b* is transcribed in wild-type, and *Or22b* RNA encodes a stable protein that shows the localization expected of an odor receptor (Figure 5D).

Or22a and *Or22b* Are Both Conserved in *Drosophila simulans*

Since we did not identify a function for *Or22b*, we considered the possibility that it has lost function over evolutionary time. In this case, the absence of premature stop codons or other mutations in *Or22b* suggests that such

(F) GFP is visualized (green) with an anti-GFP antibody.

(G) Anti-22a/b antibody labeling (red) of the sensilla.

(H) A single-unit recording from a GFP-expressing sensillum, stimulated with 2-heptanone (horizontal bar). Action potentials of two different amplitudes are visible. Frequencies of both large spikes (from the A neuron) and small spikes (from the B neuron) increase in response to the odor.

(I) Response profiles of three functional types of large s. basiconica (ab1, ab2, and ab3) from control w^{1118} flies and from green sensilla of *UAS-GFP*; *22a-GAL4* (*22a-GFP*) flies to a diagnostic set of 11 odors and the solvent control, paraffin oil (po) (n = 12; error bars indicate SEM). ab1 sensilla contain four ORNs; the other types contain two. The profile of the GFP-expressing sensilla is in reasonable agreement with that of the ab3 type.

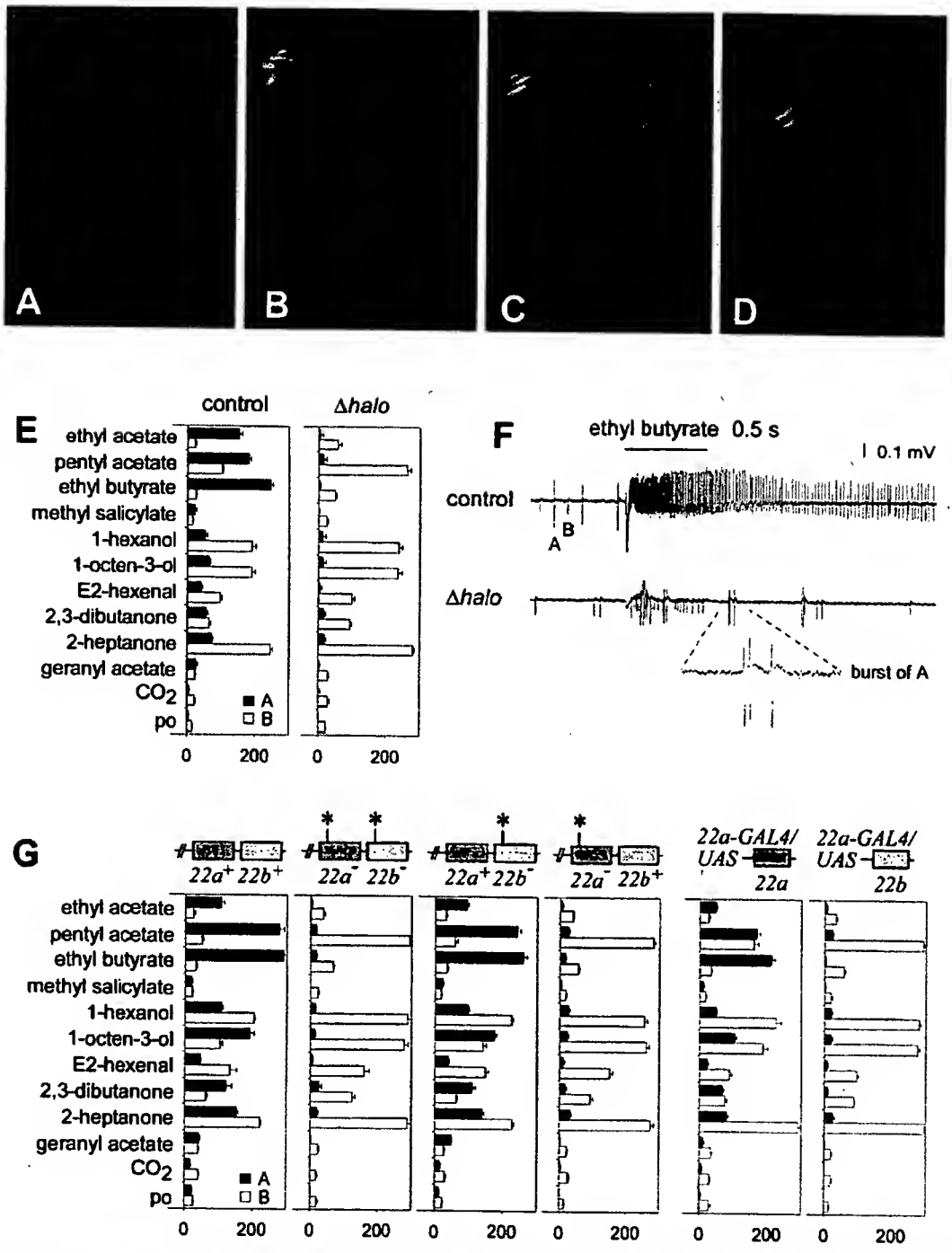

Figure 5. The Activity of the ab3A Neuron Is Abnormal in the *Δhalo* Mutant, in which *Or22a* and *Or22b* Are Deleted; Activity Is Rescued by *Or22a*, but not *Or22b*

(A–D) Immunofluorescent labeling with anti-Or22a/b antibody of (A) *Δhalo*, (B) *Δhalo*; 22a$^+$22b$^+$, (C) *Δhalo; 22a-GAL4/UAS-Or22a*, and (D) *Δhalo; 22a-GAL4/UAS-Or22b* antennae.

(E and F) Recordings from ab3 sensilla in *Δhalo* and control (w^{1118}).

(E) Response of the ab3A neuron is severely reduced to all tested odors in *Δhalo*.

(F) Traces of ethyl butyrate response showing that in the control, the A neuron (large spikes) is strongly activated by the odor, while in *Δhalo* the A neuron demonstrates very little activity. A burst from the A cell in *Δhalo* is shown expanded in the inset.

(G) Response profiles of ORNs in the ab3 sensilla from six lines containing the indicated rescue constructs (n = 10). Asterisks indicate positions of stop codons.

a loss of function would have occurred recently, perhaps following a recent duplication event. To investigate this possibility, we asked whether another *Drosophila* species, *D. simulans*, contains orthologs of *Or22a* and *Or22b*.

We identified orthologs of both *Or22a* and *Or22b* in *D. simulans* (Figure 1). These genes, which we tentatively call *DsOr22a* and *DsOr22b*, are 94% and 86% identical in amino acid sequence to their *D. melanogaster* counterparts. Neither of the *D. simulans* genes contains premature stop codons or other mutations. The evolutionary conservation of these genes, and of *Or22b* in particular, argues in favor of a functional role for *Or22b*.

We note further that the two *D. simulans* genes are tightly clustered, like their *melanogaster* counterparts: they are located within 778 bp of each other. Moreover, there is extensive sequence identity between the intergenic regions of the two species, consistent with the possibility that the two *Or* genes arose from a duplication that occurred before the two species diverged. The anti-Or22a/b antibody shows staining in the dorsomedial region of the *D. simulans* antenna, in a pattern similar to that seen in *D. melanogaster*. Physiological recordings from large basiconic sensilla in this region of the *D. simulans* antenna have revealed the presence of a sensillum whose ORNs have response spectra similar to those of ab3, consistent with a conservation of function between orthologous receptors (not shown).

Expression of an *Or* Gene Transforms the Odor Response Spectrum of an ORN In Vivo

We used the Δ*halo* mutant to investigate the relationship between *Or* expression and ORN response spectrum. One simple model is that the odor response spectra of ORNs are dictated solely by the *Or* genes they express. Alternatively, the response spectrum might be determined by an ensemble of molecules that includes not only an Or protein but also other proteins that are differentially distributed among the various sensilla and ORNs of the system. Such molecules could include odor binding proteins (OBPs) that might bind and deliver odors to the Or (Leal, 2003), other GPCRs that might heterodimerize with the Or (Jordan and Devi, 1999), or other molecules such as RAMPs (receptor activity-modifying proteins; McLatchie et al., 1998) that might modulate the ligand specificity of the Or. To address this issue, we used the ab3A neuron of the Δ*halo* mutant as a recipient for the expression of other *Or* genes.

We expressed an antennal gene, *Or47a*, under the control of *22a-GAL4* in the Δ*halo* mutant so as to drive its expression in ab3A cells. Large basiconic sensilla that contain ORNs with a response spectrum characteristic of the adjacent ab3B cell were then identified. We analyzed the response of the cell neighboring the ab3B cell and found that it responded to a subset of 11 odors tested (Figure 6A, left panel). This neighboring cell responds most strongly to pentyl acetate, followed by 2-heptanone, a pattern similar to that of the ab5B neuron (Figure 6A, right panel), which has been defined previously (De Bruyne et al., 2001). To test further the cell's similarity to ab5B, we measured its response to a panel of odors structurally related to pentyl acetate and to 3-methylthio-1-propanol, which elicits a much stronger response from ab5B than from any other characterized ORN on the antenna. We found that the neuron adjacent to ab3B in flies ectopically expressing *Or47a* again exhibits a response spectrum similar to that of ab5B and distinct from that of ab3A (Figure 6B). The simplest interpretation of all these results is that *Or47a* is expressed in ab5B and that it confers a response pattern similar to that of ab5B in the ab3A neuron when substituted for *Or22a/b*.

Having established that Or47a is capable of functioning when driven by *22a-GAL4* in a Δ*halo* background, i.e., in a cell that apparently contains no other functional receptors, we next asked whether it can function when driven by *22a-GAL4* in a wild-type background, i.e., in a cell containing at least one other functional receptor, Or22a. Accordingly, we expressed a *UAS-47a* construct under the control of *22a-GAL4* and then tested response across a broad concentration range to ethyl butyrate, which elicits a strong response from Or22a but a much weaker response from Or47a, and pentyl acetate, which elicits a strong response from Or47a but a weaker response from Or22a.

Recordings from cells designed to express both Or22a and Or47a yielded a dose-response curve for ethyl butyrate that is identical to that for cells expressing Or22a but not Or47a (Figure 6C). These results suggest that the expression of Or47a does not interfere with the expression and function of Or22a. Responses to pentyl acetate are greater in the cells expressing Or47a and Or22a than in the cells expressing Or22a alone (Figure 6C), indicating that Or47a is able to function in a cell that is also expressing Or22a. The response to pentyl acetate of cells expressing both Or47a and Or22a is lower than that in cells expressing Or47a alone; one interpretation of this finding is that Or22a, which has a moderate response to pentyl acetate, competes with Or47a, which has a strong response to pentyl acetate, and the integrated signal sent by the cell in the form of action potentials is lower than in cells expressing only Or47a. In any case, the differing results observed in the cell when stimulated with ethyl butyrate versus pentyl acetate are informative: in the former case, the expression of Or47a appears to have no effect on Or22a, indicating that the introduction of Or47a does not block expression of Or22a or inhibit the cell nonspecifically, whereas in the latter case it appears to engender a response greater than that of Or22a alone, indicating that Or47a is expressed in a form that is capable of contributing to olfactory signaling, despite the presence of another receptor.

Axonal Targeting of the eb3A Neuron Does Not Depend on *Or* Gene Expression

Studies of olfactory receptors in mammals have provided a growing body of evidence that they participate in axon guidance of ORNs toward their glomerular targets in the olfactory bulb (Mombaerts et al., 1996; Wang et al., 1998). In *Drosophila* the organization of ORN projections is similar to mammals, in that ORNs expressing the same odor receptor project to the same, topographically invariant glomeruli in the antennal lobe, the structural and functional equivalent of the vertebrate olfactory bulb (Gao et al., 2000; Vosshall et al., 2000).

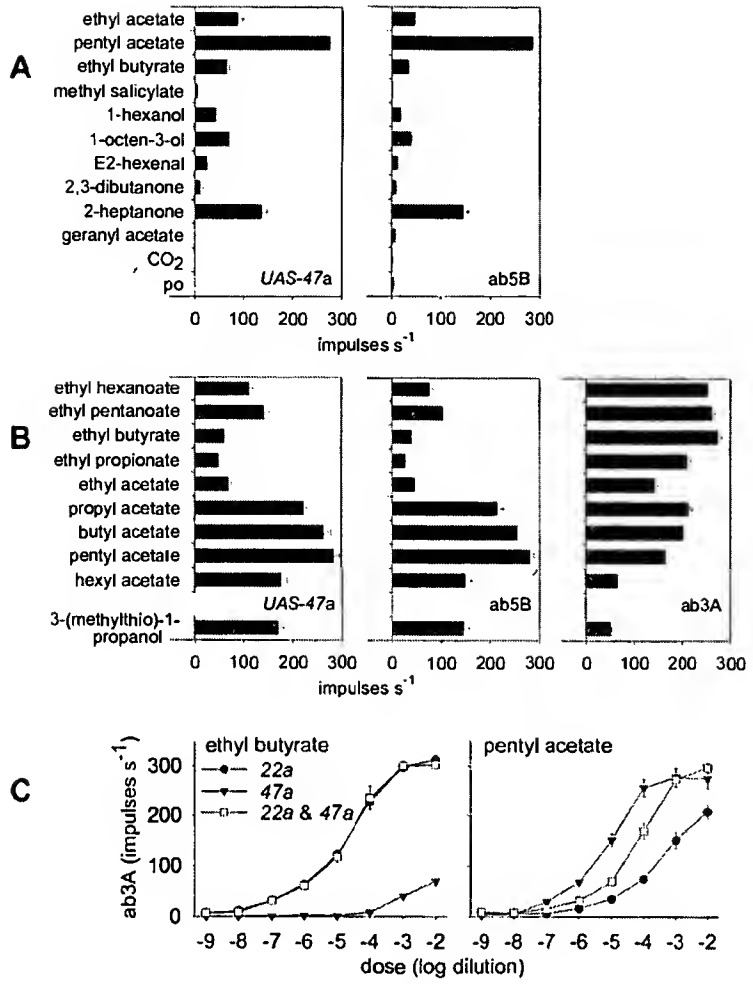

Figure 6. Ectopic Expression of *Or47a* Changes the Response Spectrum of the ab3A Neuron

(A and B) Expression of *Or47a* in the ab3A cell of a Δ*halo* mutant causes an apparent transformation to an ab5B response spectrum. Response profiles are shown for Δ*halo; 22a-GAL4/UAS-Or47a* (*UAS-47a*) flies and for ab5B neurons from w^{1118}flies. In (B), responses are also shown for ab3A from w^{1118}.

(C) Dose-response curves to ethyl butyrate and pentyl acetate of the ab3A neurons from control flies (*Or22a-GAL4*, closed circles) and from flies expressing *Or47a* in the absence of *Or22a* (Δ*halo*; *Or22a-GAL4/UAS-Or47a*, closed triangles) and in the presence of *Or22a* (*Or22a-GAL4/UAS-Or47a*, open squares). n = 6 for each genotype.

Little is known about the developmental expression of *Or* genes. Few if any *Or* genes have been systematically examined for expression throughout olfactory system development to determine whether they might play a role in ORN axon guidance or synapse formation. Moreover, expression studies are difficult to interpret because of the limited sensitivity of in situ hybridization; for example, only a small fraction of the *Gr* family of 7 transmembrane domain chemosensory receptors are detectable by in situ hybridization (Clyne et al., 2000; Scott et al., 2001). In any case, the possibility of a developmental role for *Or* genes can be rigorously addressed only by functional testing.

To determine whether *Or22a* and *Or22b* are required for axonal pathfinding of the ab3A cells, we compared their projection patterns in wild-type and in Δ*halo* mutants. Axons of the ab3A neurons were labeled using *22a-GAL4* and *UAS-GFP*. In wild-type, the ab3A neurons project to a single dorso-medial glomerulus (Figure 7A), in agreement with previous results (Vosshall et al., 2000); we have identified this glomerulus as DM2. In the mutant, the neurons project to the same glomerulus (Figure 7B), and we observed no gross abnormalities in the projections. Moreover, when *Or47a* or another odor receptor, *Or33c*, were substituted for *Or22a/b* in ab3A neurons, the axons were again observed to project to DM2 (Figures 7C and 7D). In wild-type animals, ORNs expressing *Or47a* have previously been shown to target a distinct glomerulus (Gao et al., 2000; Vosshall et al., 2000). We conclude that the targeting of the ab3A neuron to the DM2 glomerulus does not depend on normal *Or* gene expression.

Discussion

Or Proteins Localize to Dendritic Membranes of ORNs

There has been remarkably little characterization of odor receptor proteins in any organism. Attempts to raise antibodies against vertebrate ORs have been largely unsuccessful, and only two studies have reported their immunolocalization (Krieger et al., 1994; Menco et al., 1997).

We raised an antibody against the predicted N-terminal region of two closely related Or proteins, Or22a and Or22b, and used it to determine their distribution at the level of light and electron microscopy. Experiments with wild-type and mutant flies deleted for *Or22a* and *Or22b* showed that the antennal labeling is specific, and experiments with deletion mutants expressing ei-

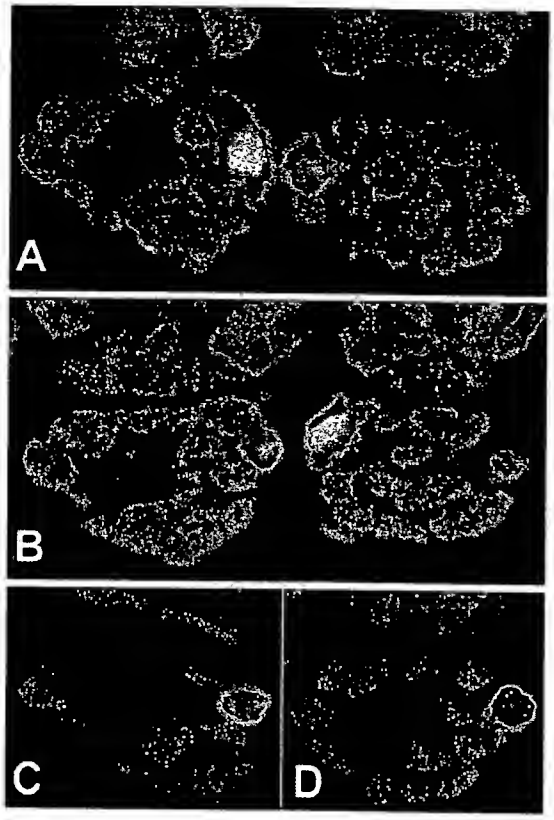

Figure 7. Targeting of ab3A Axons Does Not Depend on Or Expression

ab3A neurons send axons toward the symmetrically paired DM2 glomeruli in control *(UAS-GFP; Or22a-GAL4)* flies (A), as well as in *Δhalo* mutants (B). The DM2 glomerulus was also targeted by ORNs in which *Or47a* (C) and *Or33c* (D) were substituted for *Or22a/b* (*UAS-GFP; Δhalo; Or22a-GAL4/UAS-Or47a* or *UAS-GFP; Δhalo; Or22a-GAL4/UAS-Or33c*); panels (C) and (D) show a single antennal lobe each. The ab3A neurons were labeled with *UAS-GFP* driven by *Or22a-GAL4*, and visualized with an anti-GFP antibody (green). The antennal lobes (frontal view) were stained with monoclonal antibody nc82 (red).

ther *UAS-Or22a* or *UAS-Or22b* transgenes showed that the antibody indeed recognizes both proteins.

Immunoelectron microscopy revealed localization to the membranes of outer dendritic segments of ORNs, consistent with a role in olfactory transduction (Figures 2D and 2E). This analysis also allowed us to determine the morphological type of labeled sensilla: LB-I, a type of large basiconic sensillum.

Sexual dimorphism of receptor expression was observed by light microscopy, with almost twice as many sensilla labeled in females as males. This observation is fully consistent with the previous finding that LB-I are more abundant in females than males (Shanbhag et al., 1999).

Recently, another study described production of an antibody against the C terminus of a different member of the Or family, Or43b (Elmore and Smith, 2001). Analysis by light microscopy showed staining in the sensillum shaft, where dendrites are located. However, staining was also observed in cell bodies and axons, where we did not observe labeling.

Three Means of Mapping a Receptor to a Neuron and an Odor

Genomic analysis has identified a large family of at least 60 *Or* genes (Clyne et al., 1999a; Gao and Chess, 1999; Vosshall et al., 1999, 2000), and physiological analysis has thus far identified 22 distinct classes of receptor neurons (De Bruyne et al., 1999, 2001). An understanding of how the receptor repertoire maps to the neuronal repertoire and thence to odor space is essential to an understanding of the principles of odor coding and olfactory system development.

We have investigated the nature of this map in three ways. The first was to mark cells expressing a particular odor receptor with GFP and then to identify the marked cells by electrophysiological recording in vivo. The second approach was to mutate a receptor gene and then to determine which neuronal type was affected by physiological analysis; transgenic rescue of the mutant phenotype with a wild-type copy of the gene was used to confirm the correspondence of a particular gene with the affected neuron and its odor ligands. The third approach was based on the second, but rather than introducing a wild-type copy of the mutated gene into the mutant cell, a different receptor gene was introduced. From the odor response profile of the cell expressing the transgene, it was possible to deduce the odor response spectrum of the ectopically expressed receptor and the identity of the neuron from which it derives.

Using the first approach, we mapped the Or22a receptor to the ab3A neuron, which responds strongly to ethyl butyrate. This result was confirmed by the second approach: analysis of a mutant lacking *Or22a*, and rescue experiments using an *Or22a* transgene, also correlated Or22a with ab3A and ethyl butyrate. Moreover, Or22a was found by immunohistochemical analysis to be expressed on the dorso-medial portion of the antenna in a subset of large basiconic sensilla that contain two neurons, a description that matches ab3. We used the third approach to map the Or47a receptor to the ab5B neuron and to identify pentyl acetate as a strong ligand for this receptor.

Genetics of an Odor Receptor

The demonstration that deletion mutants lacking *Or22a* and *Or22b* are defective in odor response and that the response is restored upon introduction of an *Or22a* transgene provide direct evidence that *Drosophila Or* genes are in fact critical components of olfactory signal transduction. The *C. elegans* diacetyl receptor Odr-10 is the only chemosensory receptor previously shown to be required in vivo for normal odor detection (Sengupta et al., 1996); recently, a cluster of mouse vomeronasal receptors was shown to be required for response to pheromonal ligands (Del Punta et al., 2002), and a *Gr* gene has been shown to be essential in *Drosophila* for the response of taste neurons to the sugar trehalose (Dahanukar et al., 2001).

The effect of the deletion mutation is specific: the mutation has a profound effect on the ab3A neuron but no other ORN among the large basiconic sensilla. The

response of the ab3A cell is eliminated to all odors we have tested. Most ab3A neurons in mutant flies exhibited spontaneous electrical activity, albeit at a low rate, and thus the absence of Or22a/b did not lead to the immediate death of these neurons. We note finally that the loss of Or22a/b does not appear to lead to the de novo expression of another functional receptor, suggesting that the process of receptor gene choice does not include a receptor-mediated negative feedback mechanism.

Receptor Expression and Function: Implications for Odor Coding

A central problem in odor coding concerns the distribution of odor receptors among ORNs. A priori, there are several ways of distributing *n* receptor types among *m* functional classes of ORNs. Each receptor type could be expressed in a single ORN class, or, by contrast, in multiple, distinct ORN classes. If expressed in a single ORN class, then a receptor, R_i, could in principle be the only receptor expressed in its class, or it could be one of multiple receptors, e.g., (R_i, R_j), that are invariably coexpressed in that class. If a receptor is expressed in multiple, distinct ORN classes, then the ORN classes may be functionally distinct either because they express different combinations of receptors, e.g., (R_i, R_j) versus (R_i, R_k), or, conceivably, because each expresses the same receptor in different molecular contexts, (R_i; X) versus (R_i; Y), containing ORN class-specific differences in local OBPs, RAMPs, or heterodimerization partners, for example.

We have found that the expression of the Or22a receptor is limited to a single morphological subtype of olfactory sensillum (LB-I), a single functional type of sensillum (ab3), and a single class of ORN (ab3A). The mapping of Or22a to a single functional type of neuron argues against a model in which different neuronal classes acquire their diverse identities through the combinatorial expression of different receptors, or through the expression of a single receptor in different molecular contexts; according to these models, an individual receptor would be expressed in multiple, distinct neuronal classes.

ORNs vary in the breadth of their odor response spectrum. Physiological recordings from individual ORNs have shown that some are narrowly tuned, whereas others are broadly tuned with respect to a panel of test odors (De Bruyne et al., 1999, 2001). For example, the ab5A cell responds to only one of 47 odors tested at relatively high doses, whereas the ab3A neuron responds to a variety of esters, alcohols, ketones, and other odors of varying chain lengths. The broad tuning specificity of ORNs such as ab3A could in principle be due either to the expression of multiple receptors or to the expression of a single receptor that is broadly tuned. Our finding that deletion of *Or22a* and *Or22b* eliminates response to all tested odors, and that the full response spectrum can be rescued by *Or22a* alone, suggests that the broad response spectrum we have documented for ab3A can be attributed to one receptor, Or22a.

Our results with Or47a are also consistent with a model in which a single receptor accounts for the odor response profile of a particular ORN class. When expression of a single *Or* gene, *Or47a*, is driven in an ab3A cell lacking expression of *Or22a* and *Or22b*, Or47a confers an odor response profile like that of ab5B. These results support an interpretation in which the odor response profile of ab5B derives from the expression of a single *Or* gene, *Or47a*. Functional analysis of other *Or* genes will be required to determine the generality of these results, but they are consistent with observations made with mammalian ORNs, which are able to respond to diverse odors, apparently by virtue of expression of a single odor receptor in many (Malnic et al., 1999; Araneda et al., 2000; Bozza et al., 2002), if not all (Rawson et al., 2000; Spehr et al., 2002), cases.

Or22a, Or22b, and ab3A: Two Receptors and One Cell

While our results are consistent with a model in which Or22a is the only receptor that functions in the ab3A neuron, our data force consideration of the possibility that Or22a is not the only receptor that is expressed in the ab3A neuron. We found that both *Or22a-GAL4* and *Or22b-GAL4* drive expression of GFP in ab3 sensilla and of *rpr* in ab3A neurons. The driver constructs were designed to mimic the expression of the endogenous *Or22a* or *Or22b* genes, respectively. In each construct, *GAL4* coding sequences replace the coding sequences of the respective *Or* genes, and they include a substantial amount of DNA upstream of either *Or22a* or *Or22b* (8.2 and 10.3 kb, respectively). While it is formally possible that the expression of *Or22b-GAL4* does not mimic the expression of *Or22b* in vivo, there is no evidence for expression of *Or22b* in ORNs of the fly other than ab3A. First, in situ labeling with *Or22b* probes revealed no labeling outside the region of the antenna that contains ab3 sensilla. Second, immunolabeling showed no staining of sensilla other than LB-I in the wild-type antenna, nor the maxillary palp nor the larval olfactory organ. At the same time, it is clear that the antibody can recognize an *Or22b* product, since immunolabeling is observed in antennae lacking *Or22a* but expressing *Or22b* (Figure 5D). Moreover, *Or22b* transcripts have been amplified from the antenna by RT-PCR or found in antennal/maxillary palp cDNA libraries in multiple laboratories (this study; Clyne et al., 1999a; Vosshall et al., 1999). The simplest interpretation of all these data, taken together, is that Or22b is coexpressed with Or22a in ab3A neurons, but that Or22b is neither necessary nor sufficient for response to the odors we have used in our study.

A functional role for Or22b is nonetheless suggested by the observation that an *Or22b* ortholog is present in *D. simulans*, which diverged from *D. melanogaster* ~2.5 million years ago (Russo et al., 1995). Most important, in neither species has the gene accumulated stop codons, frameshift mutations, or deletions. One possibility is that Or22b recognizes odors not tested in our study, such as pheromones. Another possibility is that it functions only under a specific set of epigenetic, e.g., environmental, conditions.

The Cellular Context of Receptor Gene Expression

Does the odor response spectrum of a cell depend exclusively on its receptor expression or on a more complex molecular context? In the case of Or47a, the substi-

tution of Or47a for Or22a and Or22b engenders a transformation of the response spectrum from that of ab3A to that of ab5B. These results are consistent with evidence from other organisms that the odor response spectrum of an ORN depends on the odor receptor gene that it expresses (Sengupta et al., 1996; Troemel et al., 1997; Bozza et al., 2002).

ORNs in insects are intimately associated with each other in sensillar compartments. In this study we measured the activity of an ORN following genetic manipulation of its neighbor. We found that when the function of ab3A was severely compromised, either by mutation of *Or22a* and *Or22b* or by expression of the cell death gene *rpr*, the neighboring ab3B cell showed strong responses to odors. Thus, the ability of ab3B to respond to odors does not depend absolutely on the presence of a functional neighboring cell.

At the same time, however, we noted in both cases a large increase in the response of ab3B to pentyl acetate (Figures 4B and 5E). It is formally possible that this effect may arise to some extent from difficulties in counting the small ab3B spikes during intense activity of the ab3A cell. However, another interpretation is that in wild-type the activity of ab3A inhibits the response of ab3B; when this inhibition is relieved, ab3B exhibits an increased response. In some insects, neighboring ORNs in certain sensilla have been shown to respond to odors whose behavioral significance is related (Wojtasek et al., 1998; Grant et al., 1998). It seems plausible that information transmission between adjacent ORNs represents an early step in the processing of information carried by neighboring ORNs (Ochieng et al., 2002; Nikonov and Leal, 2002).

Axonal Targeting Does Not Depend on Normal *Or* Expression

An emerging body of evidence indicates that odor receptor expression is essential to normal axonal pathfinding in the vertebrate olfactory system (Mombaerts et al., 1996; Wang et al., 1998). In some but not all cases, navigation of mouse ORNs depends on odor-induced neural activity (Lin et al., 2000; Zheng et al., 2000; Zhao and Reed, 2001). Our results indicate that the ab3A neuron finds its normal glomerular target in a mutant that lacks Or22a and Or22b expression, demonstrating that these receptors are not required for targeting. Moreover, ectopic expression of either of two other receptors did not cause alterations in the targeting. We cannot exclude the possibility that Or22a and Or22b play a subtle role, or that ab3A expresses an additional *Or* gene that plays a role in pathfinding, such as *Or83b*, which is expressed widely among ORNs (Vosshall et al., 1999, 2000) and whose function is unknown. However, the simplest interpretation of our results is that ab3A finds its glomerular targets through mechanisms independent of Or expression.

One striking difference between the insect and mammalian olfactory systems is that in mammals, but not insects, ORNs are regenerated throughout adult life. Thus, pathfinding of mammalian ORNs toward their target glomeruli occurs during adult life, whereas in insects axonal pathfinding occurs only during development. Insect ORN pathfinding likely depends on a system of navigational cues that are expressed in an orchestrated temporal and spatial program. Perhaps the mammalian dependence on Ors, which are expressed both during development and during adult life (Strotmann et al., 1995), reflects the evolution of a mechanism designed to operate independently of signals that occur only transiently in development.

A second difference between insect and mammalian olfactory systems is the greater numerical complexity of mammals. The number of ORNs and glomeruli in mammals exceeds that of *Drosophila* by more than an order of magnitude, and it is likely that the mammalian olfactory system accordingly requires more information to specify the larger number of connections. Perhaps the use of extant Ors to provide developmental cues may have been the most economical means of expanding the informational content of the navigational system during evolution.

Experimental Procedures

Drosophila Stocks

Δ*halo* was kindly provided by M. Welte (Brandeis University). The absence of *Or22a* and *Or22b* genes in Δ*halo* flies was confirmed by genomic PCR analysis with five *Or22a*- or *Or22b*-specific primer combinations. The Δ*halo* deficiency also removes several other genes: some encode predicted proteins with sequence similarity to aspartic peptidase, disulfide isomerase, and regulators of chromosome condensation; another is apparently expressed in testis. Transgenic constructs were injected into w^{1118} or w^{1118}; Δ*halo* embryos and were maintained in a w^{1118} background. At least two independent lines were tested for each transgene. *UAS-mCD8-GFP; Pin/CyO* was from the *Drosophila* Stock Center (Bloomington, IN) and *D. simulans* was from the *Drosophila* Species Resource Center (Tucson, AZ). *UAS-rpr/CyO* was a gift from B. Hay (CalTech).

Immunohistochemistry

Anti-22a/b antibodies were raised in rabbits by Alpha Diagnostic (San Antonio, TX). A cysteine was added at the N terminus of the peptide and used to couple the peptide to a SulfoLink column (Pierce); antibodies were then affinity purified per the manufacturers instructions. The antibody referred to as "anti-GFP" was directed against the CD8 moiety of the CD8-GFP derivative.

For immunostaining, 14 μm frozen frontal tissue sections were collected on poly-L-lysine slides, fixed in 4% paraformaldehyde in PBS for 30 min, washed three times in PTX (PBS, 0.3% Triton-X 100), and blocked in PTX, 1% BSA for 1 hr. Tissues were incubated overnight at 4°C with primary antibodies diluted in PTX, 1% BSA, as follows: anti-22a/b, 1:75; mouse nc82 (a gift from R. Stocker), 1:20; rat anti-mCD8α (Caltag), 1:25. Secondary goat anti-rabbit Alexa 488 and 568, goat anti-mouse Alexa 568, and goat anti-rat Alexa 488 (Molecular Probes) were used at 1:250 dilution and incubated with tissues for 2 hr at RT. Both tissue sections and whole mounts, prepared as in Laissue et al. (1999), of adult brains were used for visualizing glomerular structures in antennal lobes. Images were analyzed using a BioRad 1024 laser scanning confocal microscope. Numbers of sensilla were compared using the Mann-Whitney U test.

Immunoelectron Microscopy

Drosophila heads with antennae attached were rapidly frozen by immersion into propane supercooled to −180°C and freeze-substituted in pure acetone by slowly warming the temperature from −90°C to −30°C. At −30°C, the specimens were infiltrated with Lowicryl K4M (Polysciences) and polymerization was started by UV irradiation. Sections were made with a diamond knife on a Reichert OmU2 ultramicrotome and collected on Formvar films. The postembedding protocol was as described in Laue and Steinbrecht (1997) with the primary antibody diluted 1:5 to 1:30 and the secondary antibody (goat-anti-rabbit IgG conjugated with 10 nm colloidal gold [Biocell]) diluted 1:20. Optional silver intensification (Danscher, 1981)

enlarged the gold grains to ~40 nm. Sections were stained in 2% uranyl acetate and examined in a Zeiss EM10A electron microscope.

Construction of Transgenes

To generate *22a-GAL4* and *22b-GAL4* constructs, 8,197 or 8,010 bp (for 22a-*GAL4*) and 10,287 bp (for 22b-GAL4) regions directly upstream of the corresponding predicted translational initiation codons were amplified using the Expand High Fidelity PCR system (Roche) and DNA of the P1 clone DS 05342 as a template and placed upstream of the *GAL4* gene in the pG4PN vector (C.G.W., unpublished data). The expression patterns of the two *22a-GAL4* constructs were indistinguishable. For the $22a^{+}22b^{+}$ rescue construct, an 11,993 bp fragment including 8,197 bp upstream of the *Or22a* start codon, both coding regions, and 249 bp downstream of the *Or22b* stop codon was cloned into pCaSpeR4. To generate the $22a^{-}22b^{+}$ construct, a frameshift mutation was introduced by digesting *Or22a* with BamHI and filling the 3′ ends with Klenow fragment, resulting in generation of a stop codon 7 bp downstream of the modified BamHI site. For the $22a^{+}22b^{-}$ construct, amber mutations were engineered by introducing a linker (CTAGCTAGC TAG) into the EcoRV site in *Or22b*. The $22a^{-}22b^{-}$ construct contained a combination of both mutations. For the *UAS-Or22a* construct, the coding region of Or22a beginning with the second nucleotide was PCR-amplified from P1 DNA and cloned into the pNmyc-UAST vector (C.G.W., unpublished) in-frame with the initiation codon and three copies of the myc tag coding sequence. The resulting protein has the myc tag fused in-frame to its N terminus. Expression of this protein and localization to the dendrites of neurons was confirmed by staining with anti-22a/b or with anti-myc antibody 9E10 (1:50) obtained from the Developmental Studies Hybridoma Bank (U. of Iowa). For the *UAS-Or22b* construct, a cDNA containing the entire ORF of *Or22b* was obtained by RT-PCR from antennal RNA and cloned into the pUAST vector (Brand and Perrimon, 1993). For the *UAS-Or47a* and *UAS-Or33c* constructs, the genomic region extending from immediately 5′ to the translation initiation codon to immediately 3′ of the stop codon, thus including all coding regions and introns, was PCR amplified from the corresponding P1 DNA and cloned into pUAST. The fidelity of coding regions in all constructs was verified by sequencing.

Electrophysiology

Action potentials of the ORNs in a sensillum were recorded by placing an electrode through the sensillum wall into contact with the lymph that bathes the dendrites. 5- to 15-day-old males were mounted as in Clyne et al. (1997) and De Bruyne et al. (2001). The antennal surface was observed at 1200× magnification, which allowed individual sensilla to be clearly resolved, through an Olympus BX40 microscope fitted with fluorescence optics to view GFP. For the recording electrode, a glass capillary with the tip drawn to <1 μm diameter was filled with sensillum lymph ringer (Kaissling and Thorson, 1980) and slipped over an AgCl-coated silver wire. The indifferent electrode was filled with Ephrussi and Beadle solution (Ashbumer, 1989) and was put into the head. Signal from the recording electrode was led into a $>10^{12}$ Ω input impedance amplifier (IsoDam, WPI, Sarasota, FL), fed through a 100 Hz high-pass filter into an AD-interface (GW Instruments, Somerville, MA). Recordings were analyzed offline in IGOR-Pro (WaveMetrics, Lake Oswego, OR). Only traces in which the activity of the different neurons in a sensillum could be separated on the basis of impulse amplitude (e.g., Figure 3H) were included in the data set. Responses of all neurons except ab1D (Figure 3I) were quantified from a count of the number of impulses during the 0.5 s stimulus period. The activity of the ab1D neuron was obscured by the activity of the ab1A, B, and C neurons in all but the initial and final 100 ms of the stimulus period; its firing frequency was therefore determined from a count of impulses in these two intervals. Odor stimuli were presented from Pasteur pipettes holding solutions of chemicals in paraffin oil on filter paper. Unless otherwise stated, chemicals >99.5% pure (Fluka and Sigma) were diluted to 1% v/v solutions in paraffin oil (Fluka), whereupon an aliquot of 50 μl was dropped on a 0.5 inch filter-roundel placed in the shaft of a Pasteur pipette. A pipette with CO_2 was prepared by displacing the air with CO_2 from a tank. For applying food odors, a piece of fruit wetted with a suspension of baker's yeast in warm water was placed in a Pasteur pipette. Stimuli were presented by placing the tip of a Pasteur pipette through a hole in a tube that carried an air stream (37.5 ml/s) over the fly and redirecting a flow of N_2 (3.75 ml/s) by solenoid-control through the pipette to give a 0.5 s pulse. Fresh stimulus pipettes were prepared after a maximum of three presentations; the CO_2 cartridge was renewed after single use. Each recording was taken from a different sensillum. In Figures 5G and 6C, each recording was from a different fly; in other figures they were from up to three sensilla per fly.

Cell Ablation

For cell ablation experiments, *UAS-mCD8-GFP; 22a-GAL4* or *UAS-mCD8-GFP; 22b-GAL4* lines were crossed with *UAS-rpr* flies. Initially, activity of the *rpr* gene was assessed by observing the number of GFP-expressing sensilla in the progeny of these crosses. Efficiency of the cell ablation was time dependent and reached its maximum at ~2 weeks after eclosion. Recordings were from 2-week-old flies.

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